Modification of C2,3,23,28 Functional Groups on Asiatic Acid and Evaluation of Hepatoprotective Effects

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For the development of novel hepatoprotective agents, C2, C3, C23 and C28 functional groups on asiatic acid were modified, and the prepared compounds were evaluated for their hepatoprotective effects. Among the prepared compounds, **9**, **13** and **16** showed significant hepatoprotective activities against CCl_4- and galactosamine (GalN)-induced hepatotoxicity. Especially, compound **9** showed the most significant hepatoprotective effects against GalN-induced hepatotoxicity (66.4% protection at 50 μM) and moderate hepatoprotective activities against CCl_4-induced hepatotoxicity (20.7% protection at 50 μM).

Key Words : Asiatic acid, Hepatoprotective effect, Structural modification, Hepatotoxicity

Introduction

Although acute and chronic hepatotoxicity has been recently increased, it has not been reported efficient drugs for the treatment of hepatic disease without side effects. Triterpenoids such as ursane, oleanane and lupane have been reported to exhibit hepatoprotective effect.[1-6] Among them, ursolic acid and oleanoic acid which have ursane structures showed strong hepatoprotective activity. Asiatic acid, whose structure is derived from ursane skeleton, is one of the triterpenoids isolated from *Centella Asiatica*,[7] and has moderate hepatoprotective activity in itself. Asiatic acid can also be easily prepared from hydrolysis of asiaticoside. Previously we reported modifications of C2 functional group and C11, C28, C2,3,23 or C2,23,28 functional groups on asiatic acid and evaluation of their hepatoprotective effects.[8-9] From the previous structure-activity relationship studies,[8-9] we found that simple modification of functional groups on C2, C3, C23 or C28 groups on asiatic acid greatly affected the hepatoprotective activities. It would be very interesting to prepare the modified derivatives on C2, C3, C23 and C28 functional groups on asiatic acid and evaluate hepatoprotective effects of the prepared compounds. In addition, structure-activity relationship studies of the prepared derivatives may provide valuable information for the development of novel hepatoprotective agents. In connection with previous studies, C2, C3, C23 and C28 functional groups on asiatic acid were modified and evaluated for their hepatoprotective effects.

Experimental

Material and methods. All reagents were purchased from Aldrich Chemical (www.sigma-aldrich.com) and used without further purification. Unless otherwise indicated, anhydrous solvent were distilled over CaH_2 or sodium benzophenone ketyl prior to use. Thin-layer chromatography (TLC) and column chromatography were performed with Kieselgel 60 F_{254} (Merck) and silica gel Kieselgel 60, (230-400 mesh, Merck) respectively. Compounds containing aromatic ring were visualized on TLC plates with UV light, and compounds containing oxygen were visualized on TLC plates with *p*-anisaldehyde solution. Nuclear magnetic resonance (NMR) spectra were taken on a Bruker AMX 250 MHz for ^{1}H NMR and 62.5 MHz for ^{13}C NMR, and tetramethylsilane (TMS) was used as an internal standard. Chemical shifts (δ) were recorded in ppm, and coupling constants (J) in Hz. Melting points were determined in open capillary tubes on electrothermal 1A 9100 digital melting point apparatus and were uncorrected.

Methyl 2α,3β,23-trihydroxyurs-12-ene-28-oate (2). To a stirred solution of **1** (2.6 g, 5.32 mmol) and anhydrous

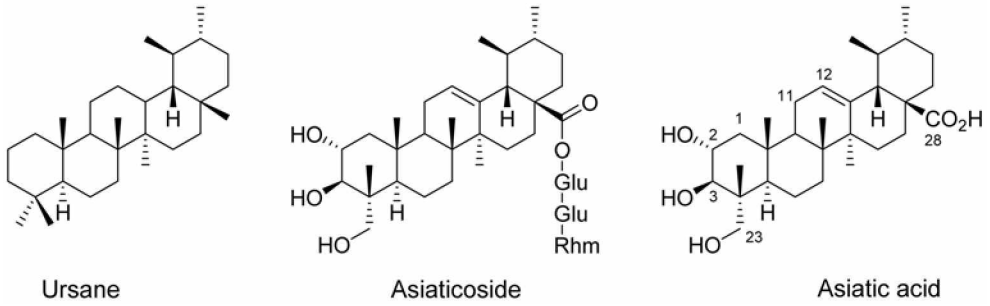


Figure 1. Structures of ursane, asiaticoside and asiatic acid.

potassium carbonate (1.84 g, 13.3 mmol) in dry DMF (20 mL) was added methyl iodide (0.66 mL, 10.64 mmol). The mixture was stirred at room temperature for 6 h. The reaction mixture was diluted with ethyl acetate (150 mL) and washed with water (50 mL×3) and saturated NaCl solution (50 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of CH_2Cl_2/MeOH (20:1, v:v) to yield a white solid (2.37 g, 88.6%).

TLC (CH_2Cl_2:MeOH=9:1, v:v). R_f=0.3. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.25 (br, 1H), 3.80-3.73 (m, 1H), 3.69 (d, J = 10.4 Hz, 1H), 3.60 (s, 3 H), 3.41, 3.47 (AB quartet, J = 9.2 Hz, 2H), 2.23 (d, J=10.8 Hz, 1H), 0.94 (d, J=6.0 Hz, 1H), 0.85 (d, J=6.4 Hz, 3H), 1.08, 1.04, 0.91, 0.75 (s, each 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C28 carbon only) δ 178.22, 138.14, 125.89.

Methyl 2α,3β,23-trimethyloxyurs-12-ene-28-oate (3). To the solution of **2** (2.37 g, 4.7 mmol) in dry tetrahydrofuran (50 mL) was added 95% sodium hydride (1.07 g, 42.7 mmol) at 0 °C and stirred for 20 min at 20 °C. To the mixture was added iodomethane (1.76 mL, 28.27 mmol) slowly and refluxed for 2 h. The solvent was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (150 mL) and washed with water (50 mL×3) and saturated NaCl solution (50 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:5, v:v) to yield a white solid (2.44 g, 95.3%).

TLC (EtOAc:*n*-hexane=1:5, v:v). R_f=0.25. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.25 (t, J=3.5 Hz, 1H), 3.60, 3.54, 3.42, 3.32 (s, each 3H), 2.23(d, J=11.2 Hz, 1H), 0.94 (d, J=6.0 Hz, 1H), 0.85 (d, J=6.4 Hz, 3H), 1.08, 0.96, 0.72, 0.65 (s, each 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C28 carbon only) δ 178.49, 138.63, 125.75.

Methyl 2α,3β,23-trimethoxyurs-11-oxo-12-ene-28-oate (4). A solution of **3** (1.5 g, 2.75 mmol) and $Na_2Cr_2O_7{\cdot}2H_2O$ (2.05 g, 6.88 mmol) in 75 mL of acetic acid was refluxed for 5 h. The mixture was cooled to 20 °C, neutralized with saturated $NaHCO_3$ to pH 7-8, diluted with ethyl acetate (150 mL) and washed with water (50 mL×3), and saturated NaCl solution (50 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:5, v:v) to yield a white solid (1.1 g, 71.6%).

TLC (EtOAc:*n*-hexane=1:5, v:v). R_f=0.15. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.61 (s, 1H), 3.61, 3.54, 3.43, 3.31 (s, each 3H), 3.23 (dd, J=12.7, 4.5 Hz, 1H), 3.14 (d, J=9.4 Hz, 1H), 3.08 (d, J=9.3 Hz, 1H), 3.05 (d, J=8.8 Hz, 1H), 2.42 (d, J=11.2 Hz, 1H), 2.37 (s, 1H), 0.97 (d, J=5.8 Hz, 3H), 0.87 (d, J=6.6 Hz, 3H), 1.30, 1.16, 0.89, 0.67 (s, each 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C11, C12, C13, C28 carbon only) δ 200.08, 177.62, 163.43, 131.00.

Methyl 2α-acetyloxy-3β,23-isopropylidenedioxyurs-12-ene-28-oate (6). To a stirred solution of **5** (2.8 g, 5.16 mmol)[8] in THF (50 mL) was added 4-dimethylaminopyridine (20 mg) at 20 °C. After stirring for 30 min, acetic anhydride (1.04 mL, 10.32 mmol) was added, and the mixture was stirred for 2 h at 20 °C. The solvent was evaporated under reduced pressure to remove THF. The residue was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:5, v:v) to yield a white solid (2.5 g, 83%).

TLC (EtOAc:*n*-hexane=1:5, v:v). R_f=0.2. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.24 (br, J = 3.4 Hz, 1H), 5.00 (m, 1H), 3.60 (s, 3H), 3.53 (d, J=10.7 Hz, 1H), 3.54, 3.48 (AB quartet, J = 10.3 Hz, 2H), 2.23 (d, J = 12.2 Hz, 1H), 2.01 (s, 3H), 0.94 (d, J = 6.0 Hz, 3H), 0.85 (d, J=6.4 Hz, 3H), 1.43, 1.41, 1.12, 1.09, 1.08, 0.73 (s, each 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C28 carbon only) δ 178.87, 138.14, 125.66.

Methyl 2α-acetyloxy-3β,23-dihydroxyurs-12-ene-28-oate (7). To the solution of **6** (9.2 g, 15.7 mmol) in THF (80 mL) was added 1 M HCl (20 mL) at 20 °C and stirred 3 h. The solvent was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (300 mL) and washed with water (100 mL×3) and saturated NaCl solution (80 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:1, v:v) to yield a white solid (6.5 g, 76%).

TLC (EtOAc:*n*-hexane=1:1, v:v). R_f=0.15. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.24 (t, J=3.6 Hz, 1H), 5.03-4.97 (m, 1 H), 3.60 (s, 3H), 2.23 (d, J=11.6 Hz, 1H), 2.02 (s, 3 H), 1.08 (d, J=6 Hz, 3H), 0.84 (d, J=6.4 Hz, 3H), 1.09, 1.07, 0.90, 0.75 (s, each 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C28 carbon only) δ 177.88, 139.11, 125.82.

Methyl 2α-acetyloxy-3β,23-hydroxyurs-12-ene-23-al-28-oate (8). A solution of pyridinium dichromate (688 mg, 1.83 mmol) and **7** (500 mg, 0.914 mmol) in dry CH_2Cl_2 (20 mL) was stirred for 3 h at 20 °C. The mixture was diluted with ethyl acetate (100 mL) and filtered to remove precipitate and washed with water (30 mL×3) and saturated NaCl solution (30 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:2, v:v) to yield a white solid (228 mg, 46%).

TLC (EtOAc:*n*-hexane=1:2, v:v). R_f=0.20. ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.40 (s, 1H), 5.25 (t, J=4 Hz, 1H), 5.05-4.99 (m, 1H), 3.60 (s, 3H), 2.24 (d, J=12 Hz, 1H), 2.08 (s, 3H), 0.94 (d, J=6 Hz, 3H), 0.85 (d, J=6.4 Hz, 3H), 1.29, 1.11, 1.09, 0.75 (s, each 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C23, C28 carbon only) δ 205.84, 177.26, 137.55, 126.39.

Methyl 2α-acetyloxyurs-3-one-12-ene-23-al-28-oate (9). A solution of pyridinium dichromate (290 mg, 0.77 mmol) and **8** (140 mg, 0.26 mmol) in dry CH_2Cl_2 (15 mL) was

stirred 3 h at 20 °C and refluxed for 2 h. The mixture was diluted with ethyl acetate (60 mL) and filtered to remove precipitate and washed with water (20 mL×3) and saturated NaCl solution (20 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:4, v:v) to yield a white solid (59 mg, 42%).

TLC (EtOAc:*n*-hexane=1:4, v:v), R_f=0.2. ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.43 (s, 1H), 5.55-5.47 (m, 1H), 5.27 (br, 1H), 3.61 (s, 3H), 2.25 (d, J=11 Hz, 1H), 2.13 (s, H), 0.94 (d, J=6.4 Hz, 3H), 0.86 (d, J=6.4 Hz, 3H), 1.34, 1.33, 1.10, 0.82 (s, each 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C3, C12, C13, C23, C28 carbon only) δ 210.36, 200.33, 177.98, 138.27, 124.89.

Methyl 2α-methyloxy-3β,23-dihydroxyurs-12-ene-28-oate (10). To the solution of **5** (2 g, 3.68 mmol) in dry THF (20 mL) was added 95% sodium hydride (0.31 g, 12.36 mmol) at 0 °C and stirred for 20 min at 20 °C. To the mixture was added methyl iodide (6 mL) slowly and refluxed for 2 h. The reaction was quenched with a drop of aqueous 1 M HCl. The mixture was concentrated under reduced pressure. To the residue was added aqueous 1 M HCl (20 mL) and THF (30 mL) at 20 °C and stirred 2 h. The solvent was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (150 mL) and washed with water (50 mL×3) and saturated NaCl solution (50 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:1, v:v) to yield a white solid (1.4 g, 78.3%).

TLC (EtOAc:*n*-hexane=1:1, v:v), R_f=0.15. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.26 (t, J=3.6 Hz, 1H), 3.32-3.24 (m, 1H), 2.24 (d, J=11.2 Hz, 3H), 0.94 (d, J=5.8 Hz, 3H), 0.85 (d, J=6.4 Hz, 3H), 3.60, 3.39, 1.07, 1.03, 0.92, 0.75 (s, each 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C28 carbon only) δ 178.56, 138.63, 125.27.

Methyl 2α-methyloxy-3β,23-diacetyloxyurs-12-ene-28-oate (11). To a stirred solution of **10** (400 mg, 0.77 mmol) in CH_2Cl_2 (10 mL) was added 4-dimethylaminopyridine (20 mg) at 20 °C. After stirring for 30 min, acetic anhydride (0.73 mL, 7.7 mmol) was added, and the mixture was stirred for 2 h at 20 °C. The solvent was evaporated under reduced pressure to remove CH_2Cl_2. The residue was diluted with ethyl acetate (50 mL) and washed with water (20 mL×2) and saturated NaCl solution (30 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:4, v:v) to yield a white solid (250 mg, 57.0%).

TLC (EtOAc:*n*-hexane=1:2, v:v), R_f=0.30. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.27 (br, 1H), 5.06 (d, J=10.1, 1H), 3.86 (d, J=11.8 Hz, 1H), 3.65 (d, J=11.6 Hz, 1H), 3.61 (s, 3H), 3.30 (s, 3H), 2.29 (s, 3H), 2.25 (d, J=12.5 Hz, 1H), 2.08 (s, 3H), 1.08, 1.03, 0.83, 0.75 (s, each 3 H), 0.94 (d, J = 6.0 Hz, 3H), 0.86 (d, J=6.5 Hz, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C3, C12, C13, C23, C28 carbon only) δ 178.47, 171.35, 167.04, 138.78, 125.43.

Methyl 2α-methyloxy-3β-hydroxyurs-12-ene-23-al-28-oate (12). A solution of pyridinium dichromate (728 mg, 1.94 mmol) and **10** (500 mg) in dry CH_2Cl_2 (20 mL) was stirred 3 h at 20 °C. The mixture was diluted with ethyl acetate (50 mL) and was filtered to remove precipitate and washed with water (20 mL×2) and saturated NaCl solution (30 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:4, v:v) to yield a white solid (220 mg, 44.1%).

TLC (EtOAc:*n*-hexane=1:4, v:v), R_f=0.15. ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.35 (s, 1H), 5.27 (t, J=3.5 Hz, 1H), 3.61 (s, 3H), 3.60 (d, J=9.4 Hz, 1H), 3.4 (s, 3H), 3.34 (m, 1H), 2.24 (d, J=11.3 Hz, 1H), 1.11, 1.09, 1.04, 0.75 (s, 3H), 0.94 (d, J=6.0 Hz, 3H), 0.85 (d, J=6.4 Hz, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C23, C28 carbon only) δ 205.85, 178.42, 138.85, 125.27.

Methyl 2α-methyloxyurs-3-one-12-ene-23-al-28-oate (13). A solution of pyridinium dichromate (395 mg, 1.05 mmol) and **12** (180 mg, 0.35 mmol) in dry CH_2Cl_2 (20 mL) was stirred 3 h at 20 °C and refluxed for 2 h. The mixture was diluted with ethyl acetate (50 mL) and filtered to remove precipitate and washed with water (20 mL × 2) and saturated NaCl solution (30 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:5, v:v) to yield a white solid (100 mg, 55.7%).

TLC (EtOAc:*n*-hexane = 1:5, v:v), R_f = 0.23. ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.42 (s, 1H), 5.28 (t, J = 3.5Hz, 1H), 3.93 (dd, J = 10.3, 6.6 Hz, 1H), 3.61 (s, 3H), 3.39 (s, 3H), 2.36 (dd, J = 13.2, 6.6 Hz, 1H), 2.26 (d, J = 11.3 Hz, 1H), 1.29, 1.21, 1.11, 0.79 (s, each 3H), 0.94 (d, J = 6.0 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C3, C12, C13, C23, C28 carbon only) d 210.02, 200.06, 178.39, 138.94, 125.09.

Methyl 2α-benzyloxy-3β,23-dihydroxyurs-12-ene-28-oate (15). To the solution of **14** (1.36 g, 2.15 mmol)[8] in THF (50 mL) was added aqueous 1 M HCl (10 mL) and stirred for 2 h at 20 °C. The solvent was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (150 mL) and washed with water (50 mL × 2) and saturated NaCl solution (60 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:2, v:v) to yield a white solid (1.15 g, 90.2%).

TLC (EtOAc:*n*-hexane = 1:2, v:v), R_f = 0.21. ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.38-7.28 (m, phenyl 5H), 5.26 (br, 1H), 4.68, 4.44 (AB quartet, J = 11.2 Hz, 2H), 3.61 (s, 3H),

3.66-3.48 (m, 3H), 3.39 (d, J = 10.7 Hz, 1H), 2.93 (s, 1H), 2.24 (d, J = 11.3 Hz, 1H), 1.08, 1.02, 0.88, 0.75 (s, each 3H), 0.94 (d, J = 6.0 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C28 carbon only) δ 178.26, 138.43, 125.27.

Methyl 2α-benzyloxy-3β,23-diacetyloxyurs-12-ene-28-oate (16). To a stirred solution of **15** (600 mg, 1.01 mmol) in THF (50 mL) was added 4-dimethylaminopyridine (20 mg) at 20 °C. After stirring for 30 min, acetic anhydride (0.57 mL, 6.07 mmol) was added, and the mixture was stirred for 2 h at 20 °C. The solvent was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (100 mL) and washed with water (30 mL × 2) and saturated NaCl solution (30 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:4, v:v) to yield a white solid (550 mg, 65.8%).

TLC (EtOAc:*n*-hexane = 1:4, v:v), R_f = 0.25, ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.35-7.25 (m, phenyl 5H), 5.27 (br, 1H), 5.08 (d, J = 10.0 Hz, 1H), 4.62 (d, J = 11.9 Hz, 1H), 4.45 (d, J = 11.9 Hz, 1H), 3.84 (d, J = 11.7 Hz, 1H), 3.66 (m, 1H), 3.61 (s, 3H), 3.56 (d, J = 11.8 Hz, 1H), 2.24 (d, J = 11.3 Hz, 1H), 2.16 (dd, J = 12.5, 4.7 Hz, 1H), 2.08, 2.02 (s, each 3H), 1.07, 1.01, 0.82, 0.75 (s, each 3H), 0.94 (d, J = 6.0 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C3, C12, C13, C23, C28, phenyl carbon only) δ 178.03, 171.06, 170.50, 138.69, 138.29, 128.24, 127.33, 127.17, 125.04.

Primary cell cultured rat hepatocytes assay. Rat hepatocytes were prepared from male Wistar rats by a collagenase perfusion technique of Berry and Friend with minor modification.[10] After 24 h, the isolated rat hepatocytes were plated, the cultured cells were exposed to a culture containing 5 mM of chloroform or glucosamine either with, or without the prepared compounds along with asiatic acid and silymarin. After 1.5 h, the activities of glutamic pyruvic transaminase (GPT) released into the culture medium were determined by the method of Reitman-Frankel.[11] All data are expressed as the mean ± SD. The evaluation of statistical significance was determined by "the one-way ANOVA" using a computerized statistical package. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

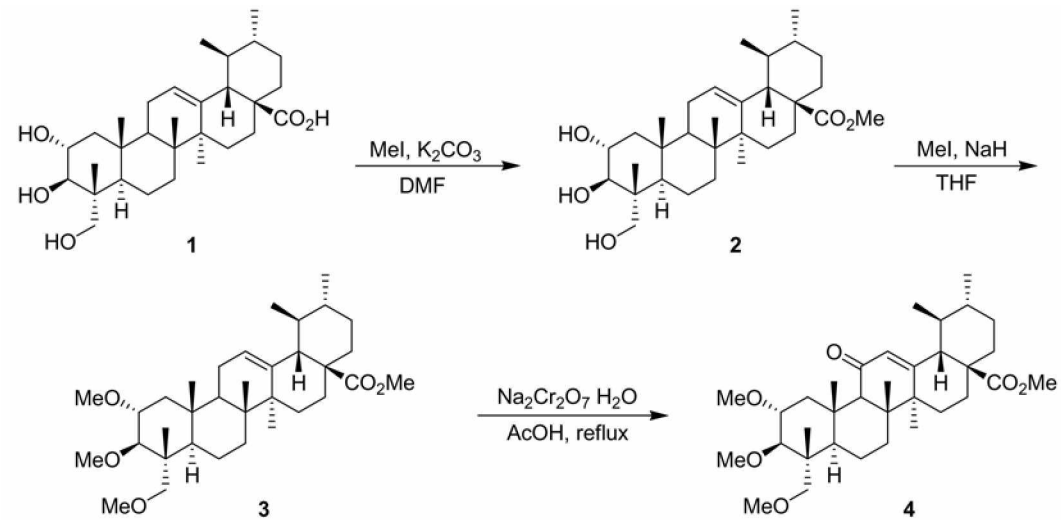
Results and Discussion

Chemistry. Methylation of asiatic acid (**1**) with methyl iodide in the presence of K_2CO_3 gave methyl ester intermediate **2** in 88.6% yield. Further methylation of **2** with methyl iodide in the presence of NaH afforded methoxy intermediate **3** in 95.3% yield, which was converted to α,β-unsaturated ketone **4** in 71.6% yield by the oxidation with $Na_2Cr_2O_7$ in the presence of acetic acid.[12] (Scheme 1)

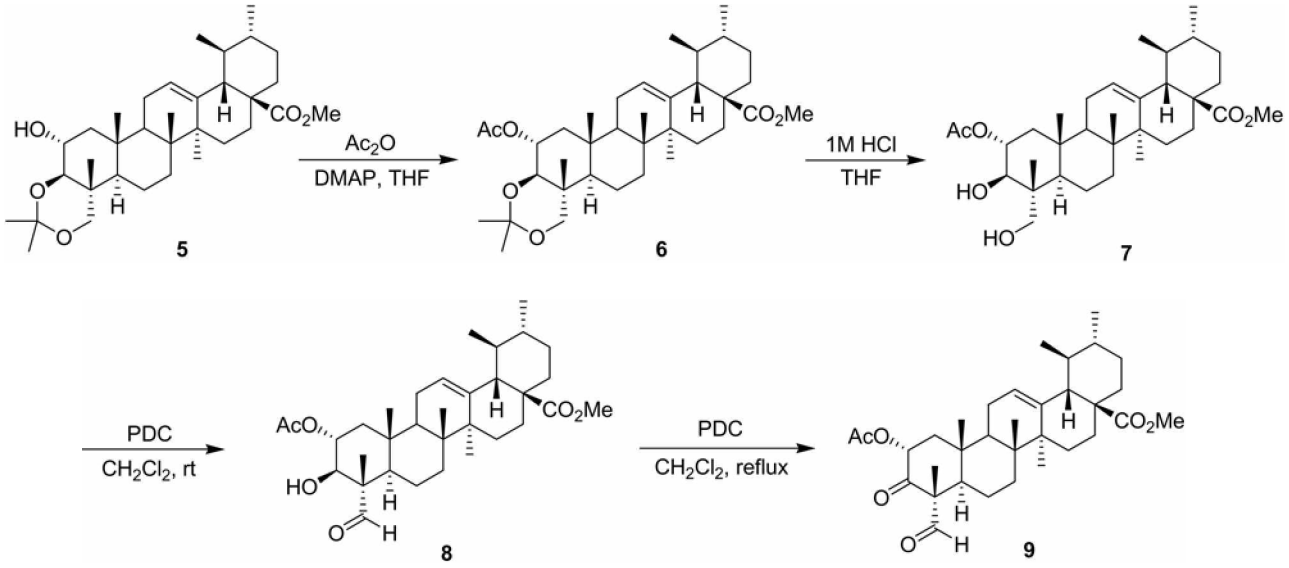
For the preparation of C2-acetoxy, C3-oxo, C23-aldehyde and C28-methyl substituted asiatate **9**, acetylation of **5**[8] with acetic anhydride gave **6** in 83% yield, which was converted to diol **7** in 76% yield by the deprotection with aqueous 1 M HCl. Concurrent oxidation of the diol in **7** would be expected directly to give β-keto aldehydes **9** which could provide various synthetic routes to modify to other compounds. But treatment of **7** with pyridinium dichromate (PDC) or pyridinium chlorochromate (PCC) gave C23-aldehyde **8** in 46% yield, which was converted to β-keto aldehyde **9** in 42% yield by the further oxidation with PDC (Scheme 2).

For the preparation of 3β,23-diacetoxy substituted compound **11**, methyl ester **5** was treated with MeI and NaH in THF, followed by deprotection with aqueous 1 M HCl to form diol compound **10** in 78.3% yield, which was treated with acetic anhydride in the presence of DMAP to afford **11** in 57.0% yield (Scheme 3).

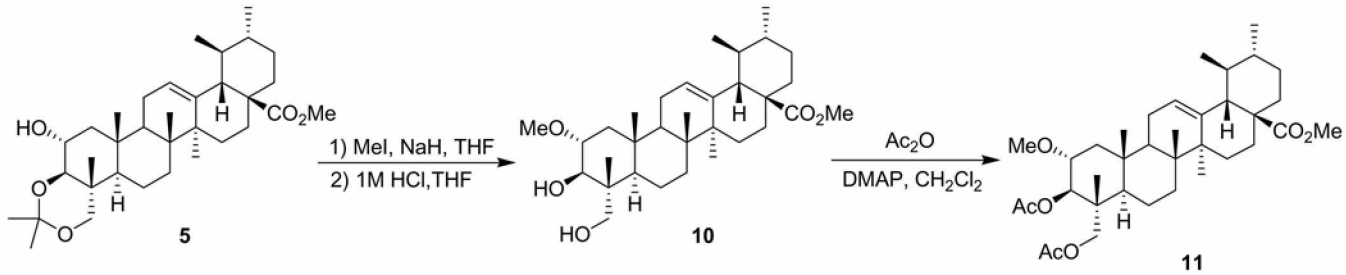
With the similar procedure described in Scheme 2, compound **13** containing 2α-methoxy, 3-oxo and 23-aldehyde functional groups was obtained in 24.6% overall yield (Scheme 4).



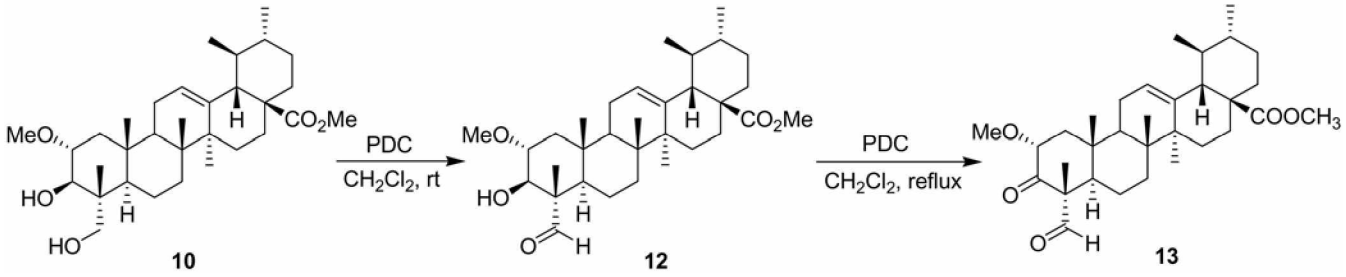
Scheme 1. Synthesis of C2,3,23-trimethoxy and C28-methyl substituted asiatic acid.



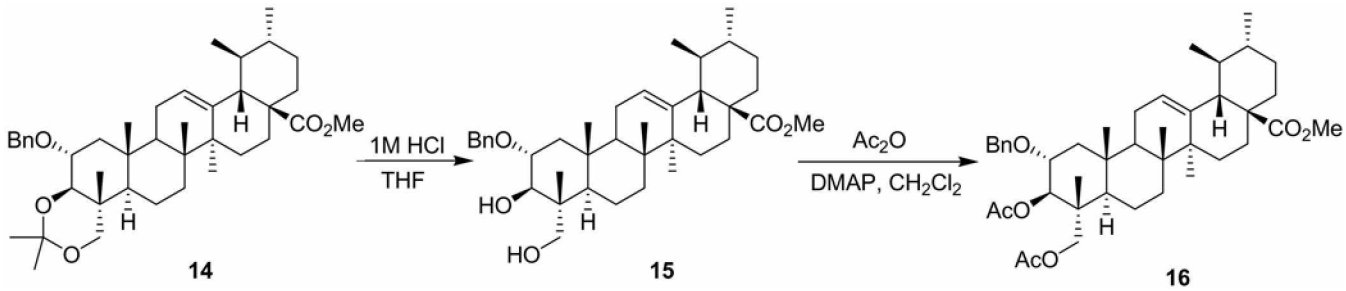
Scheme 2. Synthesis of C2-acetoxy, C3-oxo, C23-aldehyde and C28-methyl substituted asiatic acid.



Scheme 3. Synthesis of C2-methoxy, C3,23-diacetoxy and C28-methyl substituted asiatic acid.



Scheme 4. Synthesis of C2-methoxy, C3-oxo, C23-aldehyde and C28-methyl substituted asiatic acid.



Scheme 5. Synthesis of C2-benzyloxy, C3,23-diacetoxy and C28-methyl substituted asiatic acid.

For the preparation of 3β,23-diacetyloxy substituted compound **16**, **14**[8] was reacted with aqueous 1 M HCl in THF to give diol compound **15** in 90.2% yield, which was treated with acetic anhydride in the presence of DMAP to afford **16** in 65.8% yield (Scheme 5).

Pharmacology. For the evaluation of hepatoprotective

activities, hepatotoxicity was artificially induced by administration of galactosamine or CCl_4 into primary cultured rat hepatocytes. Prepared compounds were administered with different concentration, and relative glutamic pyruvic transaminase (GPT) activities were observed according to recovery of enzyme activities by Reitman-Frankel method.[11] Hepatoprotective effect was indicated as % value. (Normal GPT activity: 100%, intoxicated GPT activity: 0%). Hepatotoxicity induced by CCl_4 was reported to be due to lipid peroxide which was trichloromethyl free radical ($\cdot CCl_3$) metabolite bound with intracellular proteins and lipids by the action of cytochrome P-450 dependent mixed oxidase. Hepatotoxicity induced by galactosamine has similarities with viral hepatitis in function and formation. Galactosamine was reported to inhibit RNA and protein synthesis, which was due to alteration of amount and metabolite of uracil nucleotides in liver. Galactosamine decreased the biosynthesis of biomacromolecules related to uracil nucleotides, such as UPP-glucuronic acid, which resulted in damage of related cells and cellular organelles.

Hepatoprotective activities of the prepared compounds were evaluated. Since silymarin has been reported to show potent antihepatotoxic effects and now widely used clinically in the treatment of many liver diseases or as hepatoprotectant, silymarin was utilized as a reference compound to compare hepatoprotective activities with the tested compounds.[13,14] Silymarin was found to inhibit lipid peroxidation in rat liver microsomes and freshly isolated hepatocytes induced by pro-oxidant agents such as allyl alcohol and cumene hydroperoxide.[15] Silymarin has a very potent hepatoprotective activity (54.7% protection at 50 μM) against CCl_4-induced hepatotoxicity, but does not show any activity against galactosamine(GalN)-induced hepatotoxicity. Asiatic acid nearly does not have hepatoprotective activity against CCl_4-induced hepatoxicity (1.0% protection at 50 μM), but moderate activity against GalN-induced hepatotoxicity (23.1% protection at 50 μM).

Most of the tested compounds showed potent hepatoprotective activities. Especially, compounds **9**, **13** and **16** showed significant hepatoprotective activity against both CCl_4- (20.7%, 15.4% and 33.3% protection at 50 μM, respectively) and GalN-induced hepatotoxicity (66.4%, 52.3% and 33.6% protection at 50 μM, respectively). In addition, compound **9** showed the most significant hepatoprotective effects against GalN-induced hepatotoxicity (66.4% protection at 50 μM) and moderate hepatoprotective activities against CCl_4- induced hepatotoxicity (20.7% protection at 50 μM) (Table 1).

Modification of hydroxy group into aldehyde group at C23 position (compound **9** and **13**) dramatically increased the hepatoprotective effect (66.4% and 52.3%, respectively) against GalN-induced hepatotoxicity. Modification of methoxy group of compound **11** which has no hepatoprotective effect, into benzyloxy group at C2 position (compound **16**) increased the hepatoprotective effect (33.3% and 33.6%, respectively) against both CCl_4- and GalN-induced hepatotoxicity. It is interesting to note that simple modification of methyl group on compound **11** to benzyl group at C2 position dramatically changed the hepatoprotective effect.

Table 1. Hepatoprotective effects of the prepared compounds

Compound	CCl_4-induced	GalN-induced
	Protection (%) at 50 μM	Protection (%) at 50 μM
Asiatic acid (1)	1.0	23.1
4	12.4	15.8
9	20.7	66.4
11	NE	NE
13	15.4	52.3
16	33.3	33.6
Silymarin	54.7	NE

NE: not effective

Conclusions

In conclusion, we prepared five asiatic acid derivatives by modification of C2,3,23,28 functional groups on asiatic acid, and hepatoprotective effects of the prepared compounds were evaluated. Among them compounds **9**, **13** and **16** showed significant hepatoprotective activity against both CCl_4- and GalN-induced hepatotoxicity.

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